IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT Application of
Saville, et. al.

Group Art Unit: 1651

U.S. Serial No. 10/797,019

Examiner: Gough, Tiffany Maureen

Filed: March 11, 2004

Att. Docket No.: 95773-1439

For: ENHANCEMENT OF ENZYME ACTIVITY BY SELECTIVE PURIFICATION

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RULE 132 DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Bradley A. Saville, declare and state as follows:

1. I am currently employed by the University of Toronto. My present position and title are Professor of Chemical Engineering and Applied Chemistry.

2. I have been continuously employed since 1989 with the University of Toronto. During that time I have held the following positions, including Assistant Professor, Associate Professor, Full Professor, and Coordinator of Occupational Health and Safety. My academic and professional experience includes:

1989-1994, Assistant Professor, University of Toronto;

1994-2000, Associate Professor, University of Toronto;

2000-Present, Full Professor, University of Toronto

1991-Present, Consultant and Director, Chemical Engineering Research

Consultants Ltd.,

2001-Present, Consultant and President, 1484667 Ontario Ltd.

2003-Present, Scientific Advisor and Director, Immortazyme Company

2005-Present, Technical Advisor, BBI International and BBI Biofuels Canada

3. I am the author or co-author of many articles related to the field of biotechnology and bioprocess engineering. A list of these articles is shown in Attachment 1.

4. I am the inventor or co-inventor of many inventions related to the field of biotechnology and bioprocess engineering. A list of these patents or patent applications is shown in Attachment 2.

5. I have read and understood the present patent application. I have also read and understood the Office Actions and the cited prior art in the present patent application.

6. In my 26 April 2007 Rule 132 Declaration, I measured the enzyme activity using the well known Brix method. Brix is a measurement of the refractive index of a solution. Since the refractive index of sugars differs from that of water, the production (or consumption) of sugars can be monitored by measuring the Brix of a solution, and observing how it changes over time. Thus, the Brix values shown in Figures 1 and 2 of my 26 April 2007 declaration were measured values of the (aggregate) sugar concentration in the solution. Since sugar production is directly correlated to the activity of the enzyme, the Brix measurements in Figs. 1 and 2 are, therefore, accurate

indicators of enzyme activity.

7. I prepared the protocols and performed or supervised the following experiments to demonstrate the differences between the present invention and U.S. 2002/0020668 (Laustsen).

FIRST COMPARISON

8. The experiments for the first comparison were conducted at the University of Toronto under my direct supervision. The first comparison demonstrates that Laustsen's increased flux purification method is very different from my method that greatly enhances enzyme activity.

9. Laustsen describes the microfiltration of fermentation broth through activated carbon; Example 2 describes the typical proportions:

i) 150 kg of Savinase (alpha amylase) broth prepared according to GB 1,296,839
ii) Water (~160L, sufficient to bring total volume to 310 L)
iii) 0.3 kg of activated carbon
iv) 6.9 kg of 45% $Na_2Al_2O_4$

The process was run through a Pallsep system membrane with a 0.45 micron pore size.

For our demonstration, we scaled the quantities by a factor of 1000, i.e.,

i) 150 g of alpha amylase broth, prepared according to GB 1,296,839
ii) Water to bring total volume to 310 mL
iii) 0.3 g of activated carbon

We did not include the Al compound because, in Laustsen's words, "The microfiltration process may be further improved if an Al-product is added". Since our goal is NOT microfiltration, the Al compound was not added.

10. Description of experiments

The experiments involved passing amylase through a bed of activated carbon, monitoring the permeability (outflow rate) from the bottom of the column. Two different column arrangements were tested, one using the conventional frit with a pore size comparable to the membrane pore size used by Laustsen, and the other with filtration cloth, which has a larger pore size. The following experiments were performed, and videos were collected for all except Experiment F.:

Experiment	Enzyme volume and description	Mass of Activated Carbon	Column Frit/filtration
A	150g (137mL) raw amylase broth + water, to a total volume of 310 mL	0.30g	Standard Frit
B	1 volume of commercial amylase diluted with 9 volumes of buffer	12g	Standard Frit
C	150g raw amylase broth + water, to a total volume of 310 mL	0.30	Filter cloth
D	150g raw amylase broth + water, to a total volume of 310 mL	12g	Filter cloth
E	1 volume of commercial amylase diluted with 9 volumes of buffer	12g	Filter cloth
F (no video)	25 mL raw amylase broth diluted with water to a total volume of 250 mL	12g	Filter cloth

11. Observations from each experiment:

A: Nothing has passed through over 5 minutes; frit + carbon are completely impermeable

B: Evidence of reaction (gas release) when the diluted enzyme is initially contacted with activated carbon; a droplet is eluted from the column every ~2 seconds; approximately 30 to 40 mL eluted over 5 minutes; enzyme continues to elute after 5 minutes

C: Fast permeation for the first 15 – 20 seconds (not shown), followed by very slow permeation through filter cloth, estimated at 1 drop every ~20 seconds, or ~ 5mL per minute

D: Initially very slow permeation, then processed more quickly for several minutes, then slowed again to ~ 1 drop every 40 to 60 seconds; much more foaming observed than in experiment "C" due to reaction of carbon with protein solution

E: Initially slow, then permeates quickly, at about 30 drops every 10 seconds; eventually, the fluid is eluted as a continuous stream; ultimately, can process one column volume in a matter of seconds; significant foaming noted at top of the column

F: The liquid eluted quickly (>1 drop/second) until approximately 160mL had passed through the column. At this point the flow slowed to 1 drop every 20 seconds. Over the next 5 minutes the flow rate slowed to 1 drop every 35 seconds. The experiment was stopped at this point.

12. Conclusions:

- A raw fermentation broth, even when diluted by ~55% as specified by Laustsen, cannot flow through the column. Even with very low quantities of activated carbon (i.e., 0.3g per 310 mL of diluted fermentation broth), there is no permeation through the column and column frit. Thus, Laustsen's microfiltration process is dramatically different from the present process, which uses the combination of high levels of activated carbon, much higher dilutions, and an essentially cell-free solution. Furthermore, Laustsen applies pressure across the membrane in order to achieve the stated fluxes, whereas the claimed invention is not limited to any particular pressure.

- If the porosity restriction imposed by the column frit is removed, e.g., by replacing the column frit with filter cloth, some very limited permeation of the diluted fermentation broth is observed, but the rate drops off rapidly once the cells accumulate within the activated carbon. Operating with the high level of activated carbon specified in the present application restricts the flow even further. Thus, the fermentation broths discussed by Laustsen cannot be processed using the level of activated carbon specified by claimed invention, even if the fermentation

broth is diluted by ~55% as specified by Laustsen. If the broth is diluted to 10 times its original volume (a dilution rate specified by the claimed invention), permeation is more rapid for a while, until the cells collect on the filter cloth, after which the permeation slows dramatically again.

- There is clear evidence of a reaction (foaming, gas evolution) when the ratio of activated carbon is at the level specified in the present application, the solution is devoid of cells, and the enzyme solution has at least a 3 fold dilution.

- If a cell-free enzyme solution is used, the resulting diluted enzyme is readily permeable through the column, whether with the original column frit or with the filter cloth. If the filter cloth is used, the diluted enzyme passes through the column in a matter of seconds. This shows (1) the need for a nearly cell-free solution and/or (2) the need for a significantly diluted enzyme in order to process the solution through the high amount of activated carbon specified by the claimed invention.

SECOND COMPARISON

13. The experiments for the second comparison were conducted at a first commercial plant. I was present at the plant for three of the stages. For the stage(s) I was not present, I supervised the experiments remotely. The second comparison demonstrates that my method results in an unexpected 800% increase in enzyme activity.

14. Experimental procedures

Several hundred litres of a diluted raw enzyme solution, comprising one part raw amylase (Allzyme®, amylase from Alltech) and 14 parts of 0.05M phosphate buffer (pH 6), was prepared, then continuously passed through a bed of activated carbon for 24h. Activated carbon dust particles were removed by filtration and the resulting processed

enzyme was collected.

The processed enzyme was then used in an industrial-scale test of enzyme efficacy.

At the plant site, the processed enzyme was combined with Liquozyme® (amylase from Novozymes) at a ratio of 4 parts (by volume) purified enzyme to 1 part Liquozyme®, and added at a total flow rate of 65 mL/min to the liquefaction tank of a dry mill ethanol plant. The facility was nominally processing an average of approximately 280 USGPM of mash, with a retention time between 90 and 130 minutes in the liquefaction system. Data on sugar production during liquefaction and sugar conversion to ethanol during the subsequent fermentation step were collected. Mash flow data and related information were also collected to establish ease of processing and mash viscosity.

15. Results

The results during the trial were compared to baseline data when the plant was operating using Liquozyme®. Table 1 shows the impact on total dextrin content in the mash; during the trial with processed enzyme, total dextrins were approximately 10% higher than during normal operations with Liquozyme®, in spite of the fact that the Liquozyme® dose was reduced by 80%, along with a corresponding reduction in total "raw amylase" dose once the content of Allzyme® in the processed enzyme is also factored in.

	Dextrin concentration in mash	Brix in mash	Average % solids in slurry
Before and After Trial	22.4	26.4	34.3
During Trial	24.6	26.5	34.5
% increase	9.7%	0.3%	0.5%

Table 1: Effect on average dextrin content of mash

Figures 1 and 2 show that in spite of the lower content of raw amylase, the trial with processed enzyme provided statistically equivalent ethanol production and glucose (dextrose) conversion during fermentation compared to normal operations with Liquozyme®.

One role of alpha amylase is to sufficiently reduce the mash viscosity to ensure ease of pumping from liquefaction into fermentation, and ensure adequate heat transfer as the mash is cooled from 85°C to ~30°C prior to fermentation. Figure 3 shows that the mash flow rate into fermentation was essentially the same when using Liquozyme or when using the mixture of Liquozyme and processed enzyme.

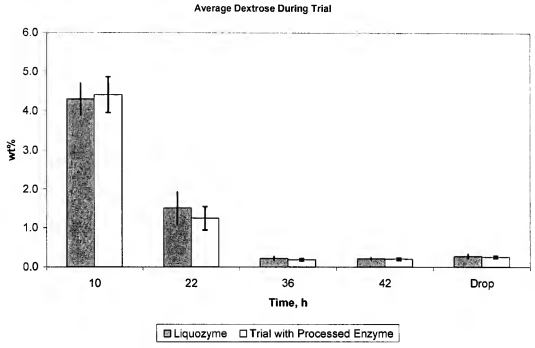


Figure 1: Dextrose Content During Fermentation

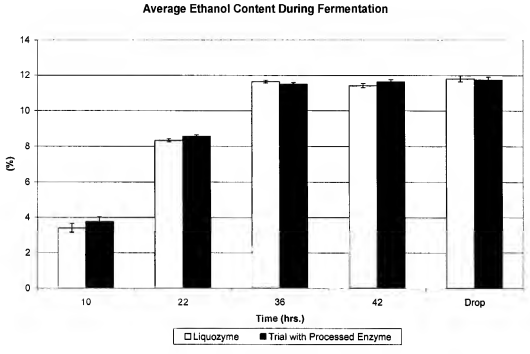


Figure 2: Weight % Ethanol Production During Fermentation

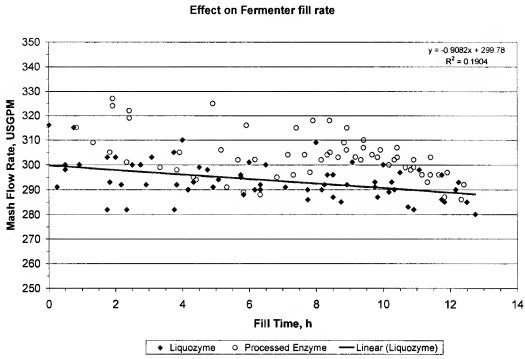


Figure 3: Impact on Mash Flow and Viscosity
(data are raw values from several fermenters)

Collectively, these data imply that the 80:20 mixture of processed enzyme with Liquozyme® gave equivalent performance when compared to an enzyme comprised of 100% Liquozyme®. This "bioequivalence" occurred in spite of the fact that the processed enzyme mixture contained only 25% of the raw alpha amylase that would be found in an enzyme solution comprised of 100% Liquozyme®. Consequently, it is apparent that the claimed process has conferred additional functionality on the protein, increasing its activity relative to unprocessed enzyme.

THIRD COMPARISON

16. The large scale experiments for the third comparison were conducted at a second commercial plant. I was present at the pant for most of the stages. For the stage(s) I was not present, I supervised the experiments remotely. This comparison also includes bench-scale experiments which demonstrate that merely diluting a purified enzyme does not result in enhanced enzyme activity. This comparison also demonstrates that my method results in an unexpected 400% increase in enzyme activity.

17. Procedures for large scale test of glucoamylase produced according to the present invention compared to merely diluted glucoamylase.

A large scale trial of modified glucoamylase was performed in a commercial scale dry grind ethanol plant. This plant uses a combination of glucoamylases added to the fermenters in a simultaneous saccharification process. The conventional 16 to 32 USG glucoamylase mixture (volumetric dose depends on the fermenter size) is comprised of 44 vol% G-Zyme 480 (Genencor) and 56 vol% Fermenzyme (Genencor), the latter of which also contains a protease and other components essential for fermentation. During this industrial trial, all of the G-Zyme 480 was replaced with modified glucoamylase, prepared as described below.

The modified glucoamylase was generally prepared as described in Example 3 of the present application, except that the volume ratio of glucoamylase to citrate buffer is 1:4 and 0.002M citrate buffer (pH 4.2) was used. The resulting mixture was fed continuously through a column containing activated carbon, and the resulting processed enzyme was collected and transported to the plant for the large scale trial.

18. Trial results:

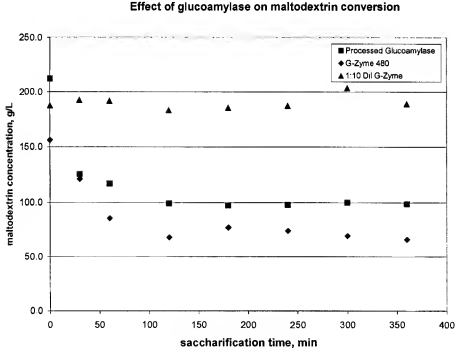
A total of 9 fermenters were dosed with a glucoamylase mixture comprised of 56 vol% Fermenzyme and 44vol% modified glucoamylase, prepared as described above. Ethanol production in each fermenter was measured, both by HPLC and by an ebulliometric method. Samples were collected from the fermenters at regular intervals and analyzed by HPLC to measure the concentrations of dextrin, maltose, glucose, ethanol, lactic acid and acetic acid. Replacement of G-Zyme 480 with modified glucoamylase led to equivalent performance compared to controls, both when assessed by HPLC and by the ebulliometric method. The average ebulliometer "burn" was 15.1 ± 0.5 (mean ± std deviation) in the 36 fermenters prior to the start of the trial, with a range from 14.3 to 16. By comparison, the average burn was 15.0 (± 0.5) in the nine trial fermenters, with a range from 13.5 to 16.5.

19. Bench scale test of glucoamylase kinetics

A trial was performed to assess the impact of dilution on the activity of glucoamylase. In these trials, one volume of G-Zyme 480 (Genencor) was diluted with 9 volumes of citrate buffer, as generally described in example 3 of the patent application. Samples of the G-Zyme 480 and diluted G-Zyme 480 were collected. The diluted G-Zyme 480 was then processed through activated carbon, as generally described in example 3. A sample of the processed enzyme was also collected. A kinetics study was then performed to measure the capacity for each enzyme (raw G-Zyme, diluted G-Zyme, and processed G-Zyme) to hydrolyze maltodextrin. In these studies, each glucoamylase sample was added to a reaction vessel initially containing 200 g/L of maltodextrin (Sigma-Aldrich). The reaction continued for 6 hours at 55°C. Samples were collected at regular intervals, and analyzed using HPLC to measure residual dextrin, along with sugar production. Figure 4 shows the dextrin conversion profiles arising from each of the three enzyme solutions tested. As shown in Figure 4, there was significant

and rapid maltodextrin conversion when raw (un-diluted, un-processed) G-Zyme 480 was used (diamonds). Diluting the G-Zyme 480 to $1/10^{th}$ of its original concentration (a 1:9 dilution) dramatically reduced its activity, leading to very little conversion of maltodextrin over the 6 hour study (triangles). In contrast, processing the 1:9 diluted G-Zyme 480 through activated carbon, as generally described in Example 3 of the present application, leads to unexpected enhancement of activity relative to the diluted, unprocessed G-Zyme 480. While the processed glucoamylase (squares) did not achieve the same conversion as the original, raw G-Zyme 480 (diamonds), the diluted enhanced enzyme was surprisingly able to convert a significant quantity of maltodextrin, in spite of the fact that it contained only $1/10^{th}$ the quantity of glucoamylase.

Figure 4



20. Bench-Scale Glucoamylase Test – Mini-fermentations

These trials were based on the procedure proposed by Allain from Novozymes (2004 FEW Workshop), wherein small-scale fermentations are tracked based on loss of mass from the system. The premise is that, as glucose is fermented to ethanol, carbon dioxide is also produced and emitted from the system, according to the reaction:

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2C_2H_5OH$$

On a mass basis, each 100 grams of glucose should produce 48.9 grams of carbon dioxide and 51.1 grams of ethanol, assuming no side reactions. Thus, a mini-fermenter should experience a decrease in mass equal to about one-half of the original substrate (glucose) mass added to the vessel. Note that this does not account for the mass gain due to dextrin hydrolysis.

While this is a relatively crude method, in that it does not account for mass losses due to evaporation of volatile components in the vessels, such as ethanol or acetaldehyde, nonetheless, it provides a reasonable representation of the SSF process.

In these trials, different glucoamylases were compared under equivalent conditions to assess their efficacy for fermentation. Each trial used a control which received all necessary reaction components except yeast and glucoamylase. Reactions were conducted in a 125mL (unjacketed) vessel. Thus, the temperature followed ambient conditions, which ranged between 23 and 27.5 °C. Reactors using mash as the substrate received ~50 mL of liquefied corn mash, 5 drops of 5% (w/v) HCl (to adjust pH to ~4.5), 0.18 g of salt, 50 μL of glucoamylase, and 0.09 g of yeast. The mass of the empty vessel was recorded, and the mass was also recorded after each item was added. The final mass, after adding (an optional) stirbar, parafilm, and elastic seal was also recorded. A

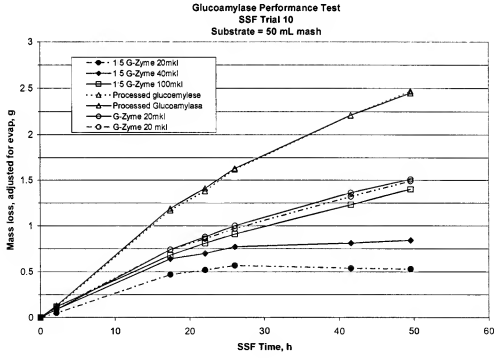
small (~2mm) hole was punched in the parafilm to allow CO_2 to escape. Several vessels were run simultaneously in a single trial, along with a control vessel (to account for evaporative losses). The mass of each vessel was recorded at several points in time, over about 50 hours. The mass loss was corrected to account for the mass loss in the control vessel (which did not contain enzyme or yeast), and plotted to compare the glucoamylases used in each trial. Within each trial, comparisons can be made because all parameters within a trial were kept constant, and each vessel would experience the same temperature history. At the conclusion of each trial, a sample was withdrawn and sent for HPLC analysis, to determine ethanol production and residual dextrin and sugars.

21. Results:

Mass loss data were obtained from a trial comparing the performance of G-Zyme 480, various doses of G-Zyme 480 diluted to 20% of its original concentration (1:5 G-Zyme), and 1:5 diluted glucoamylase processed with activated carbon. To demonstrate reproducibility, trials with processed enzyme and with raw G-Zyme 480 were completed in duplicate. Trials with diluted G-Zyme 480 were conducted with several doses. The 20 microlitre (20mkl) dose corresponds to the volumetric dose also used for raw G-Zyme 480 and for the processed glucoamylase. The 40 microlitre (40 mkl) dose represents a doubling of the enzyme dose compared to the 20 microlitre trial with diluted glucoamylase, while the 100 microlitre (100 mkl) dose of diluted enzyme would have the same total dose of glucoamylase as 20 microlitres of raw glucoamylase that had not been diluted. Thus, the G-Zyme 20 mkl trials and the 1:5 G-Zyme 100 mkl trial should give equivalent performance, unless activity has been altered due to dilution.

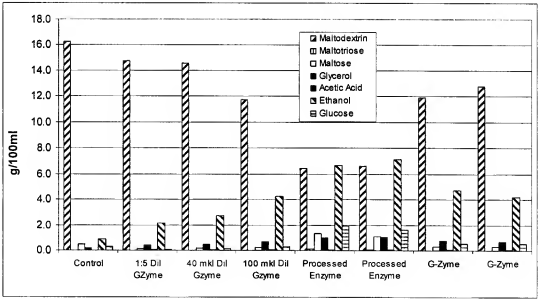
Figure 5 presents the mass loss data from these trials. In these experiments, the mass loss was clearly the least when 20 microlitres of diluted G-Zyme was used, reaching only ~0.5g. The mass loss increased to ~0.8g when the dose of diluted G-Zyme was doubled to 40 microlitres, and it increased to nearly 1.5g when the dose was increased to 100 microlitres. This latter level of mass loss was nearly equivalent to the mass loss observed with 20 microlitres of raw G-Zyme 480. Thus, there has been essentially no effect of dilution upon the activity of G-Zyme 480. Conversely, processing the diluted glucoamylase through activated carbon has significantly enhanced activity; the mass loss approached 2.5g at the conclusion of a 50 hour experiment with 20 microlitres of diluted, processed enzyme.

Figure 5:



The HPLC data obtained from analysis of the samples collected at the conclusion of the mini-fermentation experiment are presented in Figure 6. The profile from the control study (without yeast or glucoamylase) shows that there was negligible conversion of the dextrins, and minimal ethanol produced during the trial. Considering the diluted glucoamylase (1:5 Dil G-Zyme), it is apparent that the conversion of maltodextrin has increased as the dose was increased from 20 to 100 microlitres, with a corresponding increase in ethanol production. The profiles for 100 microlitres of diluted G-Zyme and 20 microlitres of raw, undiluted G-Zyme are essentially the same. By comparison, the processed enzyme has the lowest quantity of residual dextrin and the highest level of ethanol, in spite of the fact that its total glucoamylase content is 1/5th of that found in the raw glucoamylase, i.e., equivalent to the glucoamylase content of 1:5 Dil G-Zyme.

Figure 6: Effect of Glucoamylase on Final SSF Profiles



Reference:

Allain, E., "Lab-Scale Modelling of Fuel Alcohol Fermentations", presented at the 20th Fuel Ethanol Workshop, Madison, WI. (2004)

22. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: 

Bradley A. Saville

Date: 11-2 13/06